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## Expression and functional evaluation of biopharmaceuticals made in plant chloroplasts

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### Abstract

After approval of the first plant-made biopharmaceutical by FDA for human use, many protein drugs are now in clinical development. Within the last decade, significant advances have been made in expression of heterologous complex/large proteins in chloroplasts of edible plants using codon optimized human or viral genes. Furthermore, advances in quantification enable determination of *in-planta* drug dosage. Oral delivery of plastid-made biopharmaceuticals (PMB) is affordable because it eliminates prohibitively expensive fermentation, purification processes addressing major challenges of short shelf-life after cold storage. In this review, we discuss recent advances in PMBs against metabolic, inherited or infectious diseases, and also mechanisms of post-translational modifications (PTM) in order to increase our understanding of functional PMBs.

### Introduction

Biopharmaceuticals play a major role in treating various human diseases. However, they are produced in prohibitively expensive systems that require high purity and cold storage, further limited by short shelf-life. The high cost of protein drugs makes them unaffordable for most of the global population. These problems could be addressed by developing a cost-effective expression platform for production of recombinant proteins in large scale [1]. The production of pharmaceutical proteins from nuclear transgenic plants was largely abandoned by the biotech industry, mainly due to its low yield and structural heterogeneity of final products [2]. In contrast, expression of transgenes in plant chloroplast, also known as transplastomic expression, has several advantages including higher yield and more stable level of gene expression via the ability to direct transgene integration to precise sites within the chloroplast genome. Also, maternal inheritance of transgenes eliminates the escape of transgenes via pollen, offering total transgene containment when biopharmaceuticals are made in leaves [3]. Recently significant progress has been made in therapeutic protein production in chloroplasts of edible plant cells. Biopharmaceuticals produced in edible plants eliminate expensive fermentation, purification processes and cold storage/

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transportation [1,3]. Bioencapsulated protein drugs in freeze-dried plant cells are stable after several years of storage at ambient temperature [4••].

## Oral Drug Delivery

The recombinant proteins encapsulated within the plant cell wall cannot be hydrolyzed by stomach acids or other digestive enzymes and this confers a natural barrier to protect encapsulated protein drugs from degradation in the human digestive system. Once intact plant cells reach the small intestine, commensal microbes digest plant cell wall glycans, facilitating the release of expressed protein drugs. When fused with suitable tags, protein drugs cross gut epithelial cells and enter into the circulatory or immune system [5–8]. Recent studies show that oral delivery of protein to different cell types can be achieved using different transmucosal peptides fused to the N- or C-terminus of recombinant proteins [7••, Figure 1]. When cholera non-toxic B subunit (CNTB), protein transduction domain (PTD) or dendritic cell peptide (DCpep) were fused with GFP, fluorescence was widely distributed in mouse respiratory, digestive and skeletal muscle or other tissues or organs. Delivery of CTB fusion proteins across the blood-brain barrier or blood-retinal barrier was also observed in recent studies performed in mice [9••,10••]. Once delivered to the small intestine, proteins are taken up by the gut epithelial or microfold cells (M cells) before they enter the circulation system or protein drugs enter via the gut-liver axis [7••]. CTB fusion protein was detected in both human non-immune and immune modulatory cells [7••]. Recent reports also show that antimicrobial peptide (AMP), Protegrin-1 (PG-1) enters various human cell types including periodontal ligament stem cells, head and neck squamous cell carcinoma cells, Gingiva-derived mesenchymal stromal cells and adult gingival keratinocytes more efficiently than any other known cell penetrating peptides [11••].

In the last two decades, an increasing number of protective antigens against human and animal pathogens were expressed in plants [12–14]. Glucocerebrosidase made in carrot cells is the first plant-made pharmaceutical approved by U.S. Food and Drug Administration [15] to treat Gaucher's Disease. In this review, we highlight recent advances in plastid-made biopharmaceuticals (PMB), post-translational modifications (PTM) in chloroplasts and future challenges in advancing this field.

## Recombinant protein production in plant chloroplasts

### Prevention and treatment of infectious diseases

This concept involves the cloning of various vaccine antigens from bacteria, viruses, fungi or protozoa into chloroplast expression vectors and transforming them into the chloroplast genome via gene gun bombardment [3]. Once homoplasmy (transformation of all copies of chloroplast genome) is achieved, recombinant proteins are expressed at high levels - up to 70% of total leaf protein [16••,17••]. Several subunit vaccine candidates have been expressed in plant chloroplasts against various infectious pathogens [13,18]. PMBs produced against global infectious diseases include several *Mycobacterium tuberculosis* vaccine antigens, *Vibrio cholerae* non-toxin B subunit, *Bacillus anthracis* protective antigen (PA), *Yersinia pestis* F1-V antigen and *Poliomyelitis* (polio VP1) vaccine antigen with expression levels ranging from 4% to 18% of the total soluble protein [19••–23•]. Chloroplast-derived vaccine

antigens generated neutralizing antibodies upon oral boosting and conferred protection against toxin or pathogen challenges. However, vaccine antigens made in chloroplasts require injectable priming with adjuvants and can only serve as booster vaccines [19••]. Without priming, antigens can suppress immunity as described below in the immune modulation section.

In addition to subunit vaccine antigens, antimicrobial peptides have also been expressed in chloroplasts. AMPs, also called peptide antibiotics [24] are a growing class of low molecular mass oligopeptides found in many organisms with a broad range of antimicrobial activity against viruses, fungi and bacteria. Unlike antibiotics, AMPs targets lipopolysaccharides in the microbial cell membrane and induce rapid killing of pathogens. In addition to anti-bacterial effects, AMPs have been shown to possess immunomodulatory and wound healing properties [25,26••]. Both natural and synthetic antimicrobial agents have been reported so far, of which MSI-99 [26••], an analog of magainin 2, PG1 and Retrocyclin (RC101) [27•] were expressed in tobacco chloroplasts. Many human infections are biofilm associated including dental caries caused by *S. mutans*. The topical application of plant produced AMPs (PMAMP) PG1 and RC101 on tooth mimetic surface effectively inhibited biofilm formation [11••]. In addition, PMAMPs when combined with exopolysaccharide degrading enzymes (dextranase/mutanase) disrupted mature biofilms and killed *S. mutans* [11••].

### Treatment of metabolic disorders

Protein therapeutics in diabetes mellitus treatment has the highest demand as it affects over one third of adult population nationwide [28]. By utilizing transplastomic technology, expression level of complete proinsulin in plant plastids reached 50% of the total soluble protein [16••]. The massive production level in transplastomic plants facilitates scale-up of 20 million doses of insulin per acre of plant/year. Chloroplast expressed CTB-proinsulin fusion protein was efficiently assembled in plant chloroplasts, which was fully functional in animal model evaluation. Treatment of type II diabetes is also achieved using glucagon like peptide (GLP-1) that stimulates the secretion of insulin from the pancreas. Due to the extremely short half-life of GLP-1 in serum (<2 min), type II diabetes treatment has focused on dipeptidyl peptidase IV (DPP-IV) resistant analogs like exendin-4 (EX4), which has significantly longer serum half-life (~4 h). Oral delivery of plant derived EX4 fused with human transporter protein (transferrin) to mice exerted a glucose-lowering effect by stimulating insulin secretion, as well as promoting differentiation and proliferation of pancreatic  $\beta$ -cells [29]. Moreover, oral administration of plant made EX4-CTB fusion protein significantly improved glucose regulation in mice. Intriguingly, chloroplast expressed CTB-EX4 exhibited insulin stimulating effect upon oral administration without causing undesired hypoglycemia side effect [30•]. Exceptional high production level of CTB-EX4 fusion protein in transplastomic plants makes this an ideal cost-effective delivery system for type II diabetes treatment.

### Immune modulation

Hemophilia is a bleeding disorder caused by the X-linked mutation in coagulation factors. Hemophilia A and B are the most common forms of the disease affecting one among every

5,000 and 30,000 males, respectively [31]. Currently, life-time infusion of coagulation factor VIII (FVIII, for hemophilia A) or factor IX (FIX, for hemophilia B) is the standard treatment for hemophilia patients. One of the major challenges in hemophilia therapy is development of inhibitory antibodies after continuous protein replacement. This immune response to protein antigens can cause life-threatening anaphylaxis in severe cases [32]. Current clinical protocols of immune tolerance induction (ITI) require administration of high doses of blood clotting factor over a long period of time [33]. However, this clinical protocol is highly expensive and it does not guarantee the effective immune tolerance [1]. Oral delivery of plant cells expressing FIX was reported to induce immune tolerance during protein replacement therapy [34•]. Oral administration of lyophilized transplastomic plant cells expressing domains of blood coagulation factor VIII or full length FIX in chloroplasts successfully eliminated inhibitor formation in mice and dog models [4•,35•,36]. Besides hemophilia, the oral administration of the acid alpha glucosidase (GAA) epitopes expressed in plant chloroplasts [37] also eliminated antibody response in Pompe disease mouse model. Therefore, the concept of immune modulation using antigens expressed in chloroplasts has been demonstrated in several disease models.

### Post-translational modifications (PTM) in chloroplasts

PTM play an important role in maintaining the stability and bioactivity of therapeutic proteins [38]. Although PTM pathways in plant cytosol and secretory pathway have been largely elucidated, mechanisms of protein modification in chloroplasts are still poorly understood. Nevertheless, the recent proteomic studies provide further insights into chloroplast PTM [39]. Reversible phosphorylation tightly regulates the activity of protein functions in almost all aspects of cellular processes; therefore, abnormal phosphorylation is one of the major causes for a number of diseases including cancers, neurodegeneration and chronic inflammation [40,41]. Many native therapeutic proteins are phosphorylated and the phosphorylation states highly regulate protein functions [42]. Previous studies suggested that the phosphorylation of the FVa heavy chain coagulation factor increases its susceptibility to activate protein C (APC) inactivation and thereby decreases the coagulation efficiency [43]. Similarly, hyper-phosphorylation of tissue factor prevents its interaction with other hemostatic proteins and interferes with the thrombin formation [44]. The extent of phosphorylation of the vaccine carrier, monophosphoryl lipid A also affects the toxicity and immunological activity [45]. The presence of phosphorylation activity in plant chloroplasts is evidenced by the identification of phosphor-proteins localized to the chloroplast and the corresponding kinases that perform phosphorylation functions.

The sophisticated machinery of redox regulation in plant chloroplasts suggests the possibility of multimeric protein assembly in chloroplast systems via disulfide bond formation [46]. Disulfide bond formation is important to stabilize the tertiary structure and to facilitate the function of many different recombinant therapeutic proteins. The success in producing functional pharmaceutical proteins for the treatment of diabetes, blood diseases, metabolic disorders and infectious diseases in plant chloroplasts demonstrates the presence of endogenous protein disulfide transferase in chloroplasts for maintaining proper protein conformation [16•,27•,47•–49•]. RC101 expressed in chloroplast was found to be cyclized by disulfide bonds. Moreover, efficient binding of chloroplast-derived CTB fusion proteins

to the GM1 receptor requires the formation of a pentameric structure via disulfide bonds (Figure 2). In addition to the endogenous enzymes, co-expression of exogenous protein disulfide isomerase and thioredoxin showed an increase in protein folding efficiency of human serum albumin in chloroplasts [50•].

Lipidation, which is another type of PTM, refers to the covalent attachment of lipids to certain amino acids in the proteins. Lipidation of proteins facilitates subcellular targeting and protein-protein interactions [51]. The outer surface protein A (OspA) of *Borrelia burgdorferi* is an effective vaccine candidate to treat Lyme disease [52•]. Lipidation of OspA is essential for its immunogenicity and non-lipidated form did not induce protective immunity in mice. The chloroplast-derived recombinant OspA was fully functional at eliciting an immune response via the generation of protective antibodies against *burgdorferi* in mice.

## Future prospective and conclusions

Although many foreign proteins are expressed in chloroplasts, development of fully functional proteins is a significant challenge. For example, very high level expression of prokaryotic genes of bacterial origin is feasible but expression of large human or viral genes is a major challenge. Optimization of human or viral gene sequences based on the codon preference of psbA genes, from 133 sequenced chloroplast genomes, significantly increased their expression (up to 125-fold) in plant chloroplasts [53••] by improving the ribosome read-through during translation and eliminating rare codons. Drug dosage determination is currently feasible only in purified biopharmaceuticals. However, drug dosage determination in-planta has been developed for the first time using targeted proteomic quantitation by parallel reaction monitoring; when normalized with stable isotope labeled standard peptides or housekeeping proteins, targeted proteomic quantitation yielded more accurate results than quantitation by western blots, eliminating the need for protein purification before quantitation [53••]. This should facilitate further advancement of oral drug delivery of biopharmaceuticals made in plant cells. Furthermore, marker free transplastomic plants must be developed in edible crops to advance the chloroplast made biopharmaceuticals for clinical use [54]. More importantly, further studies are required to understand the post-translational modifications of foreign proteins expressed in plant chloroplasts.

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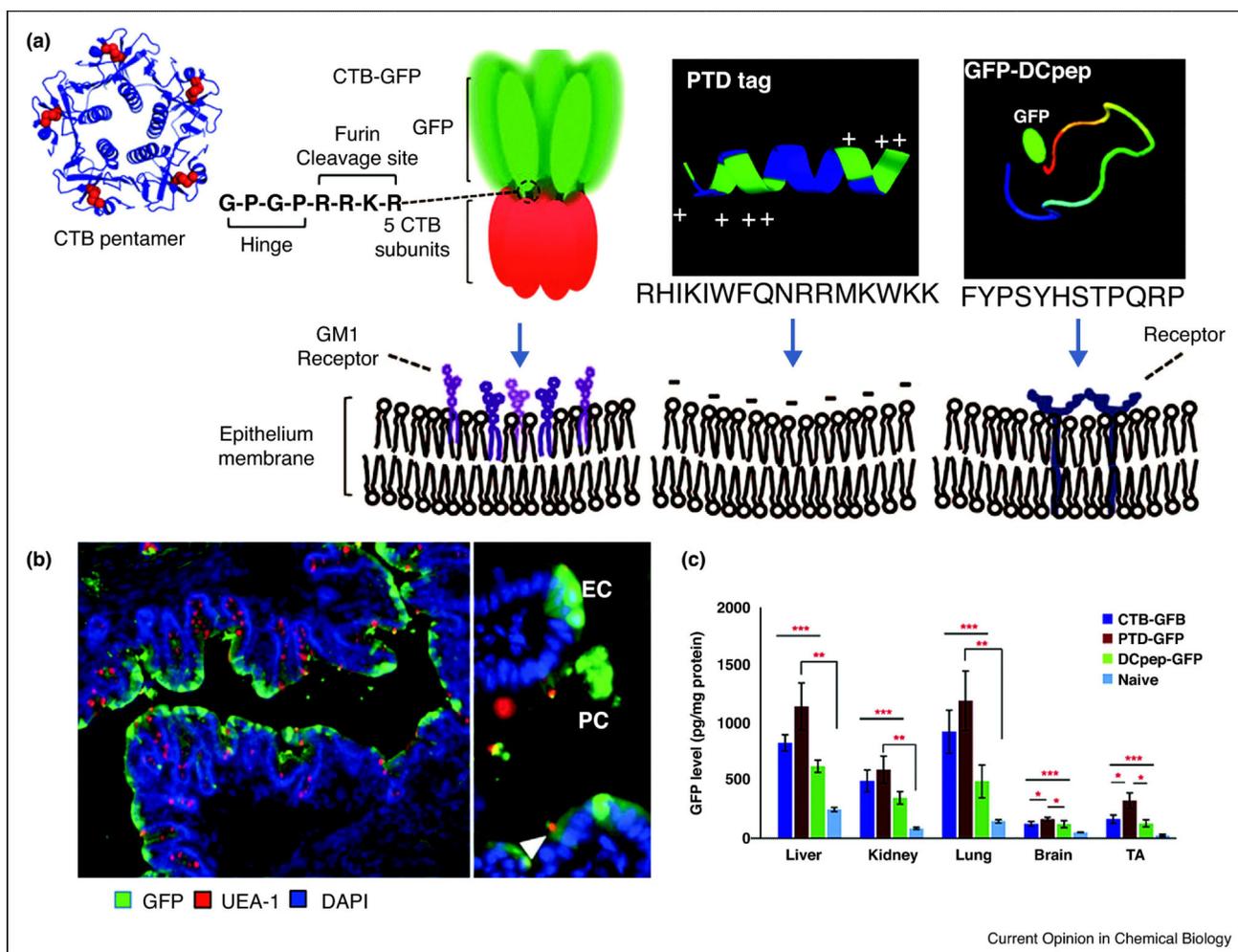
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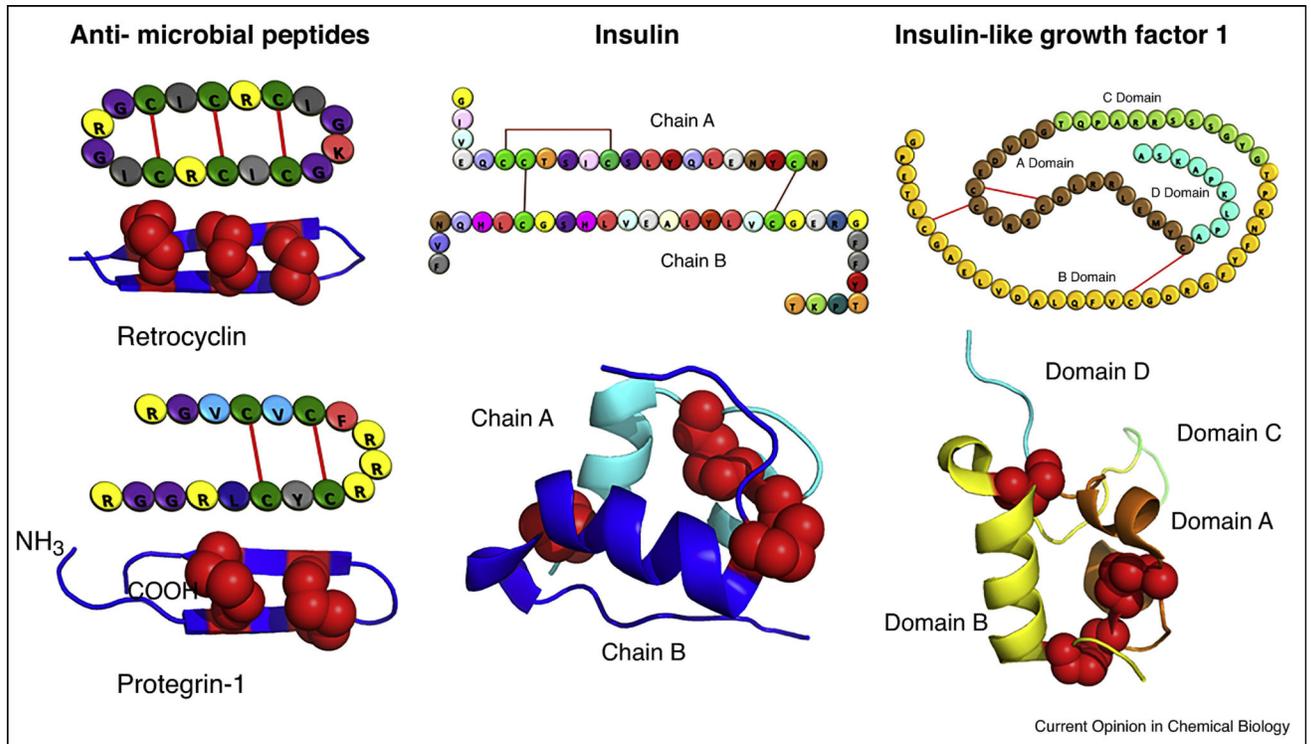
### Highlights

- Chloroplasts are ideal bioreactors for low cost production of biopharmaceuticals.
- Tags fused with protein drugs facilitate their delivery to different cell types.
- Chloroplast-derived biopharmaceuticals are properly folded and fully functional.
- Advances in drug dosage determination in-planta without purification are discussed
- Recent advances in chloroplast-made biopharmaceuticals are discussed.



**Figure 1.**

Folding and assembly of receptor-binding protein or cell-penetrating peptides expressed in plant chloroplasts for targeted drug delivery. (a) Assembly of CTB pentamer with disulfide bonds that bind to GM1 receptors and proper folding of protein transduction domain (PTD) and dendritic cell peptide (DCpep) fused to protein drugs are shown. The predicted 3D structure of CTB pentamer, PTD and Dcpep are shown. Disulfide bonds in CTB pentamer are presented as red spheres. The green color in PTD tag shows charged amino acids. (b) Transmucosal delivery of CTB-GFP through the gut epithelial cells in mouse small intestine. Small intestine sections of mice fed with lyophilized CTB-GFP cells were stained with Alexa 488 labeled anti-GFP antibody (green signal), rhodamine labeled M-cell stain (UEA-1, red signal), and nuclear stain (DAPI, blue signal). Solid arrow shows GFP+ M cells; EC, epithelial cells; PC, plant cells. (c) Biodistribution of GFP signal in different tissues of mice fed with lyophilized leaves expressing GFP fused with PTD, CTB and Dcpep tags. The figure was modified from previous publication from our lab [7•, 8].



**Figure 2.** Examples of therapeutic protein folding and modifications reported in plant chloroplasts. The predicted 3D structure of Retrocyclin, Protegrin, Insulin and Insulin-like growth factor 1 (IGF-1) are shown. Disulfide bonds are presented as red spheres. Different protein domains are labeled with different colors.